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Metabolic control of collagen synthesis

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Abstract

The extracellular matrix (ECM) is present in all tissues and crucial in maintaining normal tissue homeostasis and function. Defects in ECM synthesis and remodeling can lead to various diseases, while overproduction of ECM components can cause severe conditions like organ fibrosis and influence cancer progression and therapy resistance. Collagens are the most abundant core ECM proteins in physiological and pathological conditions and are predominantly synthesized by fibroblasts. Previous efforts to target aberrant collagen synthesis in fibroblasts by inhibiting pro-fibrotic signaling cascades have been ineffective. More recently, metabolic rewiring downstream of pro-fibrotic signaling has emerged as a critical regulator of collagen synthesis in fibroblasts. Here, we propose that targeting the metabolic pathways involved in ECM biomass generation provides a novel avenue for treating conditions characterized by excessive collagen accumulation. This review summarizes the unique metabolic challenges collagen synthesis imposes on fibroblasts and discusses how underlying metabolic networks could be exploited to create therapeutic opportunities in cancer and fibrotic disease. Finally, we provide a perspective on open questions in the field and how conceptual and technical advances will help address them to unlock novel metabolic vulnerabilities of collagen synthesis in fibroblasts and beyond.

Keywords

metabolism; fibroblasts; collagen; glycine; proline; ECM

2 Introduction

Fibroblasts are mesenchymal cells that mediate tissue homeostasis and are essential for wound healing [1]. Following tissue injury, fibroblasts are activated from a resting state through stimulation with growth factors, chief among them transforming growth factor beta 1 (TGFβ1) [2]. The binding of TGFβ1 to the heterotetramer of TGFβ receptor type I and II results in the phosphorylation of SMAD2/3 proteins, which bind to SMAD4.

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SMAD2/3/4 complexes translocate to the nucleus, where they act as transcription factors to induce the expression of pro-fibrotic genes, including alpha smooth muscle actin (αSMA, encoded by *ACTA2,* hereafter *αSMA*) and collagen I alpha 1 (encoded by *COL1A1*) [3]. In addition, TGFβ can signal non-canonically, for example, via mitogen-activated protein kinase (MAPK) or phosphoinositide 3-kinase (PI3K)/ protein kinase B (AKT)/ mammalian target of rapamycin complex 1 (mTORC1) signaling, to regulate the expression of pro-fibrotic genes [3]. As a result of TGFβ stimulation, fibroblasts acquire a myofibroblastic αSMA, high contractility, and extracellular matrix (ECM) production and secretion [2]. While the transient activation of fibroblasts supports wound healing, their chronic activation and persistent ECM production are characteristic of and drive fibrotic disease [2]. Similarly, accumulation of activated fibroblasts is observed in solid tumors [4], where they are referred to as cancer-associated fibroblasts (CAFs), supporting the notion of tumors as wounds that do not heal [5]. CAFs and their secreted ECM can promote tumor progression, metastasis, and resistance to therapy [6–8]. As a result, over the last decades, efforts in anti-cancer and anti-fibrosis therapy have often focused on the signaling cues involved in fibroblast activation, including TGFβ signaling, as reviewed elsewhere [9]. However, these approaches did not lead to the anticipated success, at least in part due to the pleiotropy of TGFβ signaling, resulting in a variety of toxicities [3].

The ECM comprises a variety of specialized proteins such as collagens, glycoproteins, and proteoglycans, as well as polysaccharides in the form of glycosaminoglycans (GAGs) [10]. As overproduction of ECM components is at the center of cancer and fibrotic disease [11], more recently, efforts for anti-cancer and anti-fibrotic therapy have shifted towards targeting the ECM directly. For example, targeting hyaluronic acid accumulation in pancreatic cancer using recombinant enzymes has emerged as a promising strategy to create therapeutic vulnerabilities in mouse models [12,13]. Still, it has not proven beneficial in clinical trials [14,15]. In addition, genetic inhibition of the synthesis of specific collagen types in pancreatic CAFs enhanced tumor progression [16]. These studies highlight the complex role of the ECM in cancer and suggest the need for novel strategies to modulate the ECM for anti-cancer therapy.

For decades, anti-cancer therapy has relied on exploiting the metabolic reprogramming associated with cancer, starting with Sidney Farber's use of anti-folates for the treatment of leukemia in children [17]. Cancer cells are highly proliferative, so these approaches often target the metabolic pathways that generate the macromolecules required to duplicate cellular biomass (Figure 1A) [18,19]. Hence, one can argue that applying the same logic to the ECM might help identify novel, targetable liabilities of ECM production. Like proliferation, ECM synthesis represents a significant demand for biomass generation, yet with requirements distinct from proliferative cells (Figure 1A). While ECM composition is tissue-specific, in general, lipids and nucleic acids are absent from ECM biomass, with proteins and polysaccharides in the form of GAGs accounting for the entire ECM dry weight [20] (Figure 1A). This suggests that the metabolic demands of macromolecule synthesis in fibroblasts engaged in ECM synthesis differ from those of highly proliferating cells. Among ECM proteins, collagens are the most abundant in the human body, representing up to 17% of the total protein content of entire mice and up to 50% of specific tissues [21] (Figure 1B). In tumors, the collagen content in the ECM can rise to almost 95% [22]

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(Figure 1B). Fibrillar collagens consist of three helically coiled polypeptide chains stabilized by hydrogen bonds between each chain, rather than hydrogen between amino acids in the same chain often found in helical proteins [23]. Each polypeptide chain contains repetitive tripeptide units with the formula Gly-X-Y, in which proline is often found in the X position and its modified version hydroxyproline in the Y position [23]. As a result, compared to proteins comprising cellular biomass, collagens within the ECM are highly enriched in the amino acids glycine and proline [24] (Figure 1C). As the smallest amino acid, glycine fits tightly into the center of the collagen triple helix but can still provide residues for hydrogen bonding to other chains [23]. Proline, with its pyrrolidine ring, provides conformational rigidity and stabilizes the collagen triple helix [25]. Glycine represents, on average, 22% of all amino acids in collagen (ranging from 9.2–28.1%), compared to approximately 7% in the average human protein; similarly, the average proline content of collagen is 15% (ranging from 8.2–22.4%), compared to less than 7% in the human proteome (Figure 1C) [24]. A similar relationship has been observed experimentally comparing fibroblast-derived ECM with all cellular proteins [24]. Thus, given collagen's unique amino acid composition, activated fibroblasts have a high demand for glycine and proline. This suggests that aberrant collagen synthesis in cancer and fibrotic disease requires fibroblasts to rewire their metabolism to fulfill these biosynthetic demands. Therefore, identifying and targeting the metabolic pathways involved in fulfilling the demands of ECM biomass generation presents a novel opportunity to develop strategies to perturb pathological collagen synthesis.

In the following sections, we will summarize recent findings on the metabolic pathways supporting collagen production in activated fibroblasts. We will focus on glycine and proline biosynthesis, the role of signaling and nutrient availability in regulating these pathways, and highlight potential therapeutic opportunities.

3 Metabolic pathways that support collagen synthesis

Fibroblasts must undergo metabolic rewiring to fulfill the unique biosynthetic demands imposed by collagen synthesis downstream of pro-fibrotic signaling cascades. Moreover, aberrant translation of collagens has high bioenergetic costs and poses unique requirements on the cellular translation machinery. Once synthesized, collagen molecules undergo a series of unique post-translational modifications (PTMs) controlled by various metabolic pathways. Collagen gene expression also involves metabolism-dependent changes in chromatin states. How activated fibroblasts meet these demands has been investigated predominantly in the context of TGFβ stimulation in embryonic and adult lung fibroblasts or by comparing CAFs to normal fibroblasts in the context of breast and pancreatic cancer. It is worth noting that fibroblasts, in general, and CAFs, in particular, are a heterogeneous population of cells but acquire a myofibroblastic state with high expression of collagens under standard culture conditions [26]. In the following, we will consider both TGFβ-stimulated fibroblasts and CAFs as activated fibroblasts and indicate differences that are reported. We will also note findings that may be specific to the tissue or tumor type studied. Most studies investigating the metabolic control of collagen synthesis have focused on collagen I, and we will highlight cases in which the concepts discussed may or may not apply to other collagen types. Many of the metabolic pathways described below have also been investigated *in vivo* in mouse models of cancer, including subcutaneous and orthoptic

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xenograft models, as well as mouse models of fibrosis, including intratracheal instillation of bleomycin, a chemotherapeutic agent that causes lung inflammation and fibrosis [27]. In this section, we will detail how intracellular metabolic pathways regulate critical steps in collagen production in activated fibroblasts.

3.1 Glycine biosynthesis

Cells can import glycine or synthesize it *de novo* from serine, which can be generated from the glycolysis intermediate 3-phosphoglycerate (3PG) [28]. In the absence of extracellular serine and glycine, TGFβ-stimulated lung fibroblasts continue to produce collagen I at high levels, suggesting that fibroblasts can synthesize glycine *de novo* to meet the glycine demands of collagen synthesis [29]. Similar observations have been made *in vivo* in the bleomycin mouse model of pulmonary fibrosis fed serine and glycine-free chow [30]. Indeed, the ability of TGFβ to upregulate collagen I protein in lung fibroblasts lacking microenvironmental serine and glycine is abrogated by the knockdown of phosphoglycerate dehydrogenase (PHGDH), the rate-limiting enzyme for serine synthesis from glycolytic carbon [29]. Consistently, pharmacologic inhibition of PHGDH is sufficient to reduce bleomycin-induced pulmonary fibrosis in mice [30]. Moreover, serine hydroxymethyltransferase 2 (SHMT2), which catalyzes the mitochondrial conversion of serine to glycine, is required for TGFβ-induced collagen I synthesis, while cytosolic SHMT1 is dispensable [29]. Both PHGDH and SHMT2, but not SHMT1, are induced by TGFβ signaling and are upregulated in fibrotic compared to healthy lungs [29,30]. TGFβ induced PHGDH and SHMT2 expression is mediated by PI3K–AKT–mTORC1 signaling and activating transcription factor 4 (ATF4) [31,32]. Consistently, genetic or pharmacological inhibition of mTORC1 or ATF4 blunts TGFβ-induced collagen I synthesis [31,32]. Thus, in response to TGFβ stimulation, fibroblasts fulfill the glycine demands of increased collagen synthesis via mTORC1/ATF4 mediated activation of serine and glycine biosynthesis.

Stable isotope tracing is conducted to evaluate the contribution of nutrients to cellular metabolites or macromolecules. In such experiments, cells are fed a stable isotope of a nutrient, often ^{13}C, whose carbons are then traced into metabolites of interest. Using the mass-to-charge shift of one unit relative to regular ^{12}C carbon, the contribution of the tracer to the metabolite of interest can be compared to other unlabeled sources by mass spectrometry. Using this technique, it has been demonstrated that TGFβ promotes the utilization of glucose-derived carbon for serine and glycine biosynthesis and that glucose-derived glycine is incorporated into collagen I [24,29,31] (Figure 2). These findings show the relevance of glucose-dependent serine/glycine biosynthesis for collagen production in fibroblasts. They also raise the question of how fibroblasts generate the high amount of the glycolytic intermediate 3PG required for serine and glycine biosynthesis. In response to TGFβ stimulation, fibroblasts increase glycolytic flux in a mTORC1-dependent but ATF4-independent fashion, increasing lactate secretion [24,29,31–33] (Figure 2). The increase in glycolytic flux is thought to be mediated by TGFβ-induced upregulation of hexokinase 2 (HK2) expression [34]. HK2 is a key enzyme that promotes glycolytic flux by phosphorylating imported glucose, thereby capturing it for intracellular use [35]. Consistently, genetic and pharmacological inhibition of HK2 abrogated TGFβ

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induced collagen I accumulation and bleomycin-induced pulmonary fibrosis in mice [34]. Similar results were obtained in mouse models of renal fibrosis [36]. Downstream of glucose phosphorylation, inhibition of pyruvate kinase (PKM) and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) attenuates renal and pulmonary fibrosis, respectively [36,37]. In addition to hexokinase, glucose import via glucose transporter 1 (GLUT1, encoded by *SLC2A1*, hereafter *GLUT1*) also controls glycolytic flux [35]. TGFβ induces *GLUT1* expression via mTORC1 and ATF4 [29,31]. As a result, TGFβ promotes glucose uptake in fibroblasts [24,37] (Figure 2), and TGFβ-induced collagen I production is dependent on glucose uptake and the presence of glucose in the environment [29,31]. Notably, glycine supplementation partially rescues the effects of glucose deprivation or mTORC1 inhibition on collagen I synthesis in lung fibroblasts [31]. Together, these findings suggest that TGFβ induced glucose uptake and glycolytic flux promote collagen production at least in part by diverting glycolytic carbon into serine and glycine biosynthesis (Figure 2).

In addition to glycolytic carbon, serine/glycine biosynthesis requires a nitrogen source for the reaction catalyzed by phosphoserine aminotransferase (PSAT1) downstream of PHGDH. Stable isotope tracing experiments in fibroblasts using α-^{15}N-glutamine show labeling of serine and glycine [24], indicating glutamine-derived glutamate as the nitrogen source. TGFβ stimulation further increases the contribution of glutamine nitrogen to the serine and glycine pool, consistent with TGFβ-induced upregulation of PSAT1 via PI3K/AKT/mTORC1 signaling [24,32,38]. PSAT1 is also expressed at higher levels in fibrotic lungs than in normal lungs [38]. Finally, genetic and pharmacological inhibition revealed that PSAT1 is required for TGFβ-induced collagen I synthesis in lung fibroblasts [38]. These data indicate that fibroblasts fulfill the high glycine demands of aberrant collagen synthesis in response to TGFβ stimulation by increasingly fueling glucose carbon and glutamine nitrogen into serine and glycine biosynthesis (Figure 2).

3.2 Proline biosynthesis

Collagen synthesis has long been known to be independent of the proline concentration in culture media [39]. The ability of fibroblasts to synthesize proline *de novo* is consistent with the fact that fibroblasts *in vitro* readily produce collagen when cultured in Eagle's medium and its modified version (DMEM), which lack proline [40]. Proline is synthesized in mitochondria from glutamate in mammalian cells via two reduction reactions. First, glutamate is reduced to gamma-glutamic semi-aldehyde (GSA) via the enzyme delta-1-pyrroline-5-carboxylate synthase (P5CS, encoded by *ALDH18A1*, hereafter *P5CS*) using the reducing equivalent nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor [41,42] (Figure 2). GSA spontaneously cycles to generate delta-1-pyrroline-5-carboxylate (P5C), which is further reduced to proline via mitochondrial pyrroline-5-carboxylate reductase (PYCR1/2) or cytosolic PYCR3 using NADH as electron donor [43] (Figure 2). TGFβ induces expression of all enzymes involved in proline biosynthesis in fibroblasts [24,38]. Similarly, breast CAFs express higher levels of *P5CS* and *PYCR1/2* compared to normal fibroblasts [44]. Notably, *P5CS* expression is upregulated in the lungs of patients with idiopathic pulmonary fibrosis (IPF) and is inversely associated with lung function [24,38]. Similarly, *PYCR1* expression is upregulated in the stroma of triple-negative breast cancer patients and associated with poor patient outcomes [44].

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Consistent with the upregulation of proline biosynthetic enzymes and collagens' high proline demands, free proline levels are higher in TGFβ stimulated fibroblasts or breast CAFs than in normal fibroblasts [24,38,44]. P5CS is required for proline biosynthesis in TGFβ-stimulated fibroblasts, breast and pancreatic CAFs [24,44,45]. While PYCR1/2 individually were dispensable for TGFβ-induced proline biosynthesis [24], PYCR1 is required for proline biosynthesis in breast CAFs [44]. Tissue-specific differences in the type of fibroblast used might explain the differential requirement of PYCR1 for proline synthesis. The study investigating proline metabolism in response to TGFβ stimulation used embryonic fibroblasts, which are likely more metabolically flexible than CAFs derived from adults with breast cancer. Moreover, growth factors other than TGFβ might contribute to acquiring a CAF state that could restrict metabolic flexibility. Importantly, collagen I synthesis in TGFβ-stimulated embryonic and lung fibroblasts and breast and pancreatic CAFs depends on P5CS and PYCR1, respectively, which is partially rescued by proline supplementation [24,38,44,45] (Figure 2). Notably, this dependency was also observed for collagen VI in breast CAFs [44], the collagen with the lowest proline content, suggesting that proline biosynthesis is a generalizable requirement for collagen synthesis in fibroblasts. Xenograft co-transplantation experiments confirmed the requirement of P5CS and PYCR1 for CAF-mediated collagen production in pancreatic and breast tumors [44,45]. Moreover, these assays also indicate that CAF PYCR1 supports breast cancer cells' tumor growth and metastatic capacity [44]. Taken together, these studies reveal a critical role of the proline biosynthetic pathway in fibroblasts' ability to produce collagen and promote disease pathology. Notably, an inhibitor of PYCR1 blunts CAF-mediated proline and collagen synthesis in culture models [44], indicating that the proline biosynthesis pathways could be targeted to alleviate collagen accumulation in tumors.

Which nutrients do activated fibroblasts use to synthesize proline *de novo*? Stable isotope tracing experiments using ^{13}C-glucose and ^{13}C-glutamine revealed that fibroblasts predominantly use glutamine carbon for proline biosynthesis, with only a minor contribution (<10%) of glucose carbon [24] (Figure 2). This is interesting, as glucose carbon contributes up to 40% to the cellular glutamate pool, from which proline is produced [24]. The reason for this disconnect remains to be investigated but may involve metabolic compartmentalization between the cytosol and mitochondria. While glutamate can be synthesized from glucose-derived alpha-ketoglutarate in both mitochondria and the cytosol, proline is synthesized from glutamate only in mitochondria (Figure 2). Glutamine utilization for proline biosynthesis is further increased in fibroblasts upon TGFβ stimulation in an SMAD4-dependent fashion [24] (Figure 2). Similarly, the contribution of glutamine carbon to the proline pool is higher in breast CAFs than in normal fibroblasts [44]. Furthermore, glutamine-derived proline is incorporated into collagen in fibroblast-derived ECM in culture and breast xenograft tumors [24,44], demonstrating that proline biosynthesis from glutamine is relevant for fibroblast collagen synthesis (Figure 2). TGFβ-stimulated fibroblasts increase glutamine uptake from the environment [24,46] (Figure 2), likely to sustain higher glutamine utilization for proline biosynthesis. Glutamine utilization for proline biosynthesis requires its conversion to glutamate in mitochondria via glutaminolysis catalyzed by the enzyme glutaminase (GLS) (Figure 2). Consistently, TGFβ induces GLS expression and activity in fibroblasts via SMAD signaling [24,38,46,47], and upregulation of collagen synthesis

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upon TGFβ stimulation is GLS-dependent [38,46,47]. Notably, genetic and pharmacological inhibition of GLS attenuates bleomycin-induced pulmonary fibrosis in mice [48]. Together, these studies highlight the role of glutamine-derived proline biosynthesis in collagen production and indicate novel opportunities for metabolic interventions to inhibit collagen accumulation in cancer and fibrotic disease.

In addition to the enzymes directly involved in glutamine utilization for proline biosynthesis, mitochondrial NAD kinase (NADK2) is required for proline biosynthesis [41,42] (Figure 2). Normal fibroblasts and pancreatic CAFs depend on NADK2 for collagen production [42,45], and *NADK2* expression is inversely correlated with lung function in patients with idiopathic pulmonary fibrosis [42]. NADK2 catalyzes the ATP-dependent phosphorylation of mitochondrial NAD(H) to NADP(H), thereby generating the NADPH required as a cofactor for P5CS [41,42] (Figure 2). Alternative activation of mitochondrial NADPH production rescued proline biosynthesis in cells with *NADK2* deletion [42], validating mitochondrial NADPH *de novo* synthesis, rather than an unknown function, as the primary mechanism by which NADK2 supports proline biosynthesis. NADK2 expression in pancreatic CAFs depends on mitophagy, and CAFs with defective mitophagy consistently cannot maintain mitochondrial NADPH levels and proline biosynthesis [45]. Moreover, mitophagy is required for collagen I production in pancreatic CAFs, which is rescued by proline supplementation [45]. These findings suggest that mitophagy supports proline and collagen I synthesis in pancreatic CAFs by maintaining NADK2 expression and, thus, mitochondrial NADPH *de novo* synthesis. Notably, the recently reported crystal structure of NADK2 has revealed important sites of regulation as well as dissimilarity from cytosolic NADK involved in lipid metabolism [49,50], suggesting that the structure-based design of selective NADK2 inhibitors is a novel strategy for developing anti-fibrotic and anti-cancer therapy approaches.

3.3 Bioenergetic and other demands of collagen translation

When stimulated with TGFβ, fibroblasts increase their translation rate [24,46], likely to support the aberrant synthesis of ECM proteins. Such elevated translation rates impose high bioenergetic demands in the form of ATP required for tRNA charging and GTP synthesis consumed during elongation. In response to TGFβ stimulation, fibroblasts increase ATP production from both glycolysis and oxidative phosphorylation (OXPHOS); however, only the increase in OXPHOS-mediated ATP production correlated with the elevated translation rates over time [24]. These findings suggest that the high rates of OXPHOS observed in TGFβ-stimulated fibroblasts support collagen synthesis at least in part through sustaining protein translation.

The nutrients oxidized to generate the NADH needed to stimulate OXPHOS and, thus, ATP synthesis, have been investigated by stable isotope tracing. These experiments revealed that TGFβ stimulation promotes the oxidation of glucose and glutamine-derived carbon in the mitochondrial tricarboxylic acid (TCA) cycle [24] (Figure 2). For complete glucose oxidation, the end product of glycolysis, pyruvate, is imported into mitochondria. There, the pyruvate dehydrogenase (PDH) complex catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA, generating NADH and linking glycolysis to the TCA cycle. TGFβ-stimulated

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fibroblasts and breast CAFs show higher activity of the PDH complex and, thus, higher contribution of glucose carbon to acetyl-CoA and the TCA cycle [24,44,51] (Figure 2). High PDH activity in breast CAFs is maintained by repression of its negative regulator, pyruvate dehydrogenase kinase 2 (PDK2) [44]. Conversely, inhibition of another PDK isoform, PDK1, suppresses pulmonary fibrosis in mice [52]. PDK1 inhibition phenocopies effects observed by fibroblast-specific deletion of hypoxia-inducible factor 1 alpha (HIF-1α, encoded by *Hif1a*) in the same model [52]. HIF-1α activity suppresses pyruvate oxidation in mitochondria by inducing *PDK1* [53], suggesting increased mitochondrial oxidative metabolism in lung fibroblasts impairs fibrosis development. The discrepancy related to the role of mitochondrial pyruvate oxidation in breast CAFs and lung fibroblasts might be explained by the inflammation occurring before the onset of fibrosis in the bleomycin mouse model of pulmonary fibrosis. In the harsh environment of inflammation, suppression of mitochondrial oxidation and, thus, the potential of generating damaging reactive oxygen species might benefit fibroblast survival. In contrast, the upregulation of mitochondrial pyruvate oxidation during the fibrotic phase could help fulfill the bioenergetic needs of aberrant collagen synthesis.

If stable isotope tracing experiments are conducted with time as a variable and with experimentally determined or estimated metabolite uptake and secretion rates, flux through metabolic pathways can be inferred. Metabolic flux analysis demonstrated a 2.5-fold increase in flux from glutamine towards alpha-ketoglutarate and, thus, the TCA cycle in fibroblasts in response to TGFβ stimulation [24,46] (Figure 2). This increase in flux matches elevated glutamine uptake rates and flux through GLS following TGFβ stimulation [24]. In line with GLS, increased glutamine utilization in the TCA cycle following TGFβ stimulation is SMAD-dependent [24,46]. The use of glutamine carbon to support the TCA cycle is a form of anaplerosis, which refers to replenishing TCA cycle intermediates used to support anabolic metabolism [54]. Glutamine anaplerosis in fibroblasts is likely a consequence of citrate export from mitochondria through the citrate transport protein, as the contribution of glucose carbon beyond citrate towards later TCA cycle intermediates drops substantially [24]. Despite high glutamine anaplerosis rates following TGFβ stimulation, glutamine carbon use in the TCA cycle does not support TGFβ-induced OXPHOS [38], suggesting that it is relevant for processes other than supporting the bioenergetic needs for collagen translation. In addition, these findings indicate that high GLS activity in activated fibroblasts supports collagen production primarily through mechanisms other than anaplerosis, such as proline biosynthesis.

In addition to glucose and glutamine, cells can oxidize fatty acids to support OXPHOS. Transcriptomic profiling revealed impairments in fatty acid oxidation in TGFβ-stimulated fibroblasts and across multiple fibrosis mouse models *in vivo* [55,56]. Inhibition of fatty acid oxidation promotes the accumulation of collagen I and other ECM proteins in dermal fibroblasts, while its activation reduces collagen synthesis [55,56]. In contrast, enhancing fatty acid oxidation promotes collagen I degradation in dermal fibroblasts, dependent on lysosomal activity and fatty acid uptake through the transporter CD36 [56]. The mechanisms by which fatty acid oxidation regulates collagen synthesis and degradation remain to be investigated. These studies suggest that glucose and glutamine are the preferred fuels for fibroblasts to support aberrant collagen synthesis.

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During translation, proline is incorporated much more slowly than other amino acids into the growing polypeptide chain [57], making proline-rich proteins, including collagens, prone to ribosome stalling [44,58]. The conserved translation elongation factor eIF5A is specifically required to promote the translation of polyproline motifs, as well as of tripeptide sequences that combine proline with glycine, such as those found in collagens [59]. eIF5A contains the modified amino acid hypusine essential to its function, and inhibition of eIF5A hypusination impairs collagen I synthesis [60]. These findings indicate that fibroblasts producing collagen must fulfill additional metabolic requirements such as eIF5A hypusination to support collagen translation due to its high proline content (Figure 2). How fibroblasts generate sufficient hypusinated eIF5A for efficient collagen translation is an open question but may involve the metabolism of polyamines [61], enigmatic metabolites with unknown roles in collagen biology.

3.4 Metabolic control of collagen stability through post-translational modifications

Once synthesized, collagen molecules undergo extensive posttranslational modification in the endoplasmic reticulum (ER), including prolyl- and lysine hydroxylation (Figure 2) [23]. Hydroxylation of proline residues in the 4-position further stabilizes the helical structure of collagen chains [25]. In contrast, inadequately hydroxylated collagen molecules will undergo lysosomal degradation before they are secreted. Prolyl 4-hydroxylases (P4Hs) catalyze 4-hydroxyproline formation on collagen and are part of the alpha-ketoglutarate, oxygen, ascorbate, and iron-dependent family of dioxygenases, similar to those that hydroxylate hypoxia-inducible factors (HIF-α) [25]. Collagen P4Hs differ from those for HIF-α in that they are located in the ER and have a lower affinity for oxygen [62], indicating that they can hydroxylate collagen in low-oxygen environments. Since oxygen tension is generally lower in most tissues than ambient oxygen, this mechanism is thought to ensure proper collagen stability across physiological oxygen ranges. In addition, alpha-ketoglutarate availability has been shown to regulate collagen I and III stability [46] (Figure 2). Fibroblasts increase alpha-ketoglutarate levels through glutaminolysis [24,46]. Consistently, glutaminase inhibition decreases collagen I and III stability [46]. Thus, glutaminolysis regulates collagen stability through alpha-ketoglutarate, in addition to its role in supporting collagen translation through proline biosynthesis (Figure 2). In contrast, the “oncometabolite” D-2-hydroxyglutarate, which is generated through mutations in isocitrate dehydrogenase 1 and acts as an inhibitor of alpha-ketoglutarate-dependent dioxygenases, blocks prolyl-hydroxylation of collagen I and IV [63]. Moreover, ascorbate is essential for the secretion of functional collagen through its requirement in proline hydroxylation [64]. Ascorbate is the ionized, active form of ascorbic acid (Vitamin C), and given its role in collagen production by enabling proline hydroxylation, it is not surprising that deficiency in dietary Vitamin C causes scurvy, a disease of defective collagen synthesis [65].

3.5 Metabolic control of collagen gene expression

Metabolic pathways also influence collagen gene expression by regulating the availability of substrates and cofactors for chromatin-modifying enzymes (Figure 2). Acetylation of lysine residues at histones 3 and 4 (H3K3/4ac) is associated with actively expressed genes and mediated by histone acetyltransferases, including EP300 using nucleo-cytosolic acetyl-CoA as an acetyl donor [66]. Acetyl-CoA is generated in mitochondria from glycolytic

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pyruvate via the PDH complex but cannot pass through the inner mitochondrial membrane. To transport acetyl-CoA into the nucleo-cytosolic compartment, it is first condensed with oxaloacetate to citrate, then exported through the citrate transport protein in exchange for malate. In the cytosol or nucleus, citrate is converted back to acetyl-CoA by ATP citrate lyase (ACLY), linking glucose metabolism to histone acetylation [67].

The role of chromatin modifications in regulating collagen expression has been investigated in breast CAFs, TGFβ-stimulated cardiac fibroblasts and myofibroblasts derived from patients with palm fibrosis. Compared to normal fibroblasts, breast CAFs have hyperacetylated histone 3 globally and at lysine 27 (H3K27ac) at collagen gene loci, which depended on EP300 activity [44]. Similarly, the promoters and enhancers of several collagen genes are enriched for H3K27ac in myofibroblasts in an EP300-dependent fashion [68] (Figure 2). Consistently, collagen expression requires EP300 activity [44,68]. Notably, the proline metabolic enzyme *PYCR1* is also enriched for H3K27ac in breast CAFs, and EP300 regulates its expression [44,68] (Figure 2). TGFβ-induced *COL1A1* expression in cardiac fibroblasts and cardiac fibrosis depends on ACLY [69]. Breast CAFs derive acetyl-CoA from citrate via ACLY, and consistently, *COL1A1* and *PYCR1* expression in CAFs is ACLY-dependent [44]. The increase in glucose-derived acetyl-CoA in breast CAFs is mediated by higher activity of the PDH complex [44] (Figure 2). These findings suggest that CAFs rewire glucose metabolism to promote collagen gene expression and that glucose metabolism and proline and collagen biosynthesis are linked epigenetically via ACLY and EP300 (Figure 2). In support of this, metabolic perturbations that reduce TCA cycle flux and, thus, citrate levels impair H3K27ac at the *Col1a1* I locus and the expression of most collagen genes in fibroblasts, which is restored by supplementation of alternative TCA cycle substrates [51].

In contrast to acetylation, trimethylation of H3 at lysine 27 (H3K27me3) is associated with gene repression. As a result, histone demethylases promote gene expression by removal of repressive chromatin marks [66]. Like prolyl hydroxylases, histone demethylases belong to the family of alpha-ketoglutarate, oxygen, ascorbate, and iron-dependent dioxygenases. By regulating the availability of alpha-ketoglutarate, glutamine availability and glutaminolysis regulate H3K27me3 and the expression of several collagens in fibroblasts [51,70] (Figure 2). Consistently, TGFβ-induced glutaminolysis and the resulting increase in alpha-ketoglutarate levels support *COL1A1* expression at least in part by altering histone methylation [24,71] (Figure 2). This phenotype is mimicked by metabolic interventions in fibroblasts that elevate the alpha-ketoglutarate to succinate ratio [71], which serves as a measure of effective alpha-ketoglutarate available for dioxygenases.

The methylation of histones and DNA depends on the methyl donor S-adenosyl methionine (SAM). Hence, metabolic reactions that deplete the cellular SAM pool can potentially regulate gene expression by reducing DNA- and histone methyltransferase activity [66]. By transferring a methyl group from SAM to nicotinamide, the enzyme nicotinamide N-methyltransferase (NNMT) mediates DNA and histone hypomethylation in fibroblasts [72] (Figure 2). NNMT is upregulated in ovarian CAFs and omental metastasis-associated fibroblasts and promotes collagen gene expression through epigenetic remodeling [72]. Notably, an inhibitor of NNMT has been developed and shown efficacy in a mouse model of

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ovarian cancer [72], suggesting that NNMT-mediated epigenetic rewiring in CAFs could be exploited for anti-cancer therapy.

3.6 Metabolic networks that promote collagen synthesis in activated fibroblasts

In the above sections, we have detailed the metabolic rewiring in TGFβ-stimulated fibroblasts or CAFs required to fulfill the epigenetic, biosynthetic, bioenergetic, translational, and post-translational demands of elevated collagen synthesis. These individual metabolic reactions do not happen in isolation and must be integrated into metabolic networks that allow activated fibroblasts to allocate nutrients appropriately and meet all their specific demands. We proposed the following model (Figure 2): first, DNA- and histone methylation are suppressed through NNMT activity, which depletes the methyl-donor SAM. Repressive DNA and histone methylation marks are removed by increased activity of histone demethylases through elevated availability of glutamine-derived alpha-ketoglutarate. Increased glycolytic carbon flux into the TCA cycle and export of citrate from mitochondria results in elevated nucleo-cytosolic acetyl-CoA synthesis for histone acetylation. Altogether, these metabolic changes create a more accessible chromatin state permissive for transcription factors such as SMADs to promote the expression of collagens and other pro-fibrotic genes (Figure 2).

Second, metabolic flux analysis demonstrates that TGFβ promotes glutamine uptake, glutaminolysis, and glutamine flux into the TCA cycle by 2.5-fold but stimulates glutamine flux into proline by 7-fold [24]. This raises the question of how fibroblasts decide whether to allocate glutamine-derived glutamate for anaplerosis or proline biosynthesis. As described above, proline biosynthesis requires the mitochondrial redox equivalents NADPH and NADH [43]. Using genetic and pharmacological tools to modulate redox metabolism in fibroblasts and cancer cells, it was demonstrated that perturbations resulting in a decreased mitochondrial NADH/NAD^+ ratio reduce proline levels, while those leading to elevated mitochondrial NADH/NAD^+ increase proline levels [24,73,74]. Thus, electron accumulation in the form of NADH, specifically in mitochondria, drives flux from glutamine into proline biosynthesis. As a result, it has been proposed that flux through the proline biosynthetic pathway, but not proline itself, acts as a mitochondrial redox “vent,” relieving cells of extra electrons in the form of NADH [24]. This also allows regeneration of the electron acceptor NAD^+ required for cell proliferation [75]. In support of these conclusions, inhibition of proline biosynthesis under conditions of NADH accumulation leads to redox stress and impaired proliferation, even in the presence of proline [24,73,74]. Furthermore, cells increase metabolic flux towards other “vents” that allow NAD^+ regeneration when proline biosynthesis is inhibited, such as pyruvate to lactate [24]. We consider two mechanisms by which accumulation of mitochondrial NADH promotes proline biosynthesis (Figure 2): mitochondrial NADH could stimulate the reaction catalyzed by PYCR1 directly via mass-action [74], or it may be converted to NADPH by NADK2, which would promote the rate-limiting reaction for proline biosynthesis catalyzed by P5CS via mass-action [41,42] (Figure 2). While there is a large agreement between cancer and fibroblast literature regarding the “vent” function of the proline biosynthetic pathway, it is worth noting that NADH accumulation resulting from ETC inhibition increases proline levels in cancer cells, but decreases proline levels in fibroblasts [24,73]. Why these differences exist remains

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unclear but may involve the high demands of proline for translation in fibroblasts that are not as pronounced in cancer cells. While cancer cells predominantly secrete accumulating proline [73,74], proline utilization for fibroblast collagen translation comes with additional biosynthetic and bioenergetic demands that cells under ETC inhibition cannot fulfill. This includes serine/glycine biosynthesis and ATP generation, both limited under ETC inhibition [24,76]. In addition, ATP may be limiting for the reactions catalyzed by NADK2 and P5CS. We therefore propose that the mitochondrial redox and bioenergetic state regulate proline biosynthesis in fibroblasts (Figure 2).

Third, as glutamine oxidation does not significantly contribute to mitochondrial respiration, mitochondrial NADH accumulation appears to be driven by pyruvate oxidation. Thus, we propose that pyruvate oxidation via the PDH complex drives glutamine flux into proline biosynthesis (Figure 2). We further suggest that NADH utilization for proline biosynthesis maintains high rates of pyruvate oxidation and TCA cycle flux, which are inhibited by mitochondrial NADH accumulation. These fluxes then serve as a source of alpha-ketoglutarate and intermediates for non-essential amino acid (NEAA) synthesis required for tRNA-charging and translation [51]. This concept suggests that glutamine-derived anaplerosis plays a significant role in collagen production by supporting alpha-ketoglutarate and NEAA synthesis for collagen gene expression, translation, and post-translational modification. Glutamine's importance in NEAA synthesis for collagen translation is further highlighted by its role as a nitrogen donor for serine biosynthesis. Conversely, we conclude that glucose's significant roles in collagen synthesis are to support serine/glycine biosynthesis, to produce citrate as a source of acetyl-CoA, and to generate NADH that drives ATP synthesis and glutamine flux into proline biosynthesis, thereby supporting collagen gene expression and translation.

Finally, during glycine synthesis, SHMT2 transfers one-carbon units from serine to folate-derived tetrahydrofolate (THF), which is regenerated by meTHF dehydrogenase 2 (MTHFD2) in a reaction that reduces $NADP^+$ to NADPH. Given that these reactions occur in mitochondria, the site of proline biosynthesis, it is tempting to speculate that glycine and proline biosynthesis are linked through mitochondrial NADPH [77] (Figure 2). High flux through the serine biosynthetic pathway also elevates the abundance of alpha-ketoglutarate [78], which is required for collagen prolyl hydroxylation. This suggests that serine/glycine biosynthesis has additional, indirect links to collagen synthesis. In summary, while we have already learned much about how individual nutrients and metabolic pathways contribute to collagen synthesis, the above studies and proposed models highlight a need to investigate how these pathways act on a network level to support the metabolic demands of activated fibroblasts.

4 The metabolic environment impacts collagen synthesis

In the above sections, we discussed how the uptake and utilization of glucose and glutamine, the major extracellular nutrients for cells, allow activated fibroblasts to fulfill the metabolic demands of increased collagen synthesis. These conclusions predominantly stem from studies conducted under standard cell culture conditions. These conditions feature supraphysiological levels of glucose and glutamine designed to optimize cell proliferation

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[40]. In contrast, physiological levels of these nutrients are substantially lower and can drop further in healing wounds, fibrotic tissue, and tumors due to altered vascularization and blood perfusion [79–84]. In contrast, other metabolites not commonly found in cell culture media, such as lactate, are present in blood and accumulate under pathological conditions [84–86]. In addition, oxygen tension is markedly reduced in tumors and wounds [87,88]. Thus, how fibroblasts regulate collagen synthesis in the context of physiologically relevant environmental nutrient and metabolite levels is an important area of investigation. In the following sections, we will describe how the availability of glucose, glutamine, lactate, and oxygen individually impacts collagen synthesis in fibroblasts. We will also discuss how fibroblasts integrate these environmental cues to regulate metabolic pathways involved in collagen production.

4.1 Glutamine availability

In the absence of glutamine, collagen I levels in fibroblasts drop substantially [38,39], suggesting that fibroblasts depend on extracellular glutamine for collagen synthesis. In pancreatic tumor interstitial fluid, glutamine concentration can drop to as little as 0.4 mM, with various xenograft tumor cores having glutamine concentrations of 0.1–0.2 mM [80,82]. Thus, glutamine levels are substantially lower in tumors than in standard cell culture media (2–4 mM), suggesting that low glutamine conditions better model the metabolic conditions CAFs experience in the tumor microenvironment. Under such conditions, TGFβ-induced collagen synthesis in fibroblasts is glutamine-dependent, with TGFβ treatment unable to upregulate collagen I levels starting at 0.4 mM glutamine [51]. While proline biosynthesis is one of the primary ways fibroblasts use glutamine [24], impaired collagen I synthesis in low glutamine conditions is not caused by a lack of proline [51]. Instead, glutamine dependency for collagen I synthesis in TGFβ-stimulated fibroblasts is caused by an inability to maintain the TCA cycle [51], suggesting that TGFβ makes anaplerosis glutamine-dependent. As discussed, glutamine anaplerosis is not required to support OXPHOS in TGFβ-stimulated fibroblasts [38]. Instead, it is necessary to maintain the charging state of tRNA iso-decoders for NEAAs [51]. Thus, under low glutamine conditions, NEAA tRNA charging becomes limiting to sustaining the high rates of translation induced by TGFβ stimulation.

Interestingly, unstimulated fibroblasts can maintain collagen I production in low-glutamine environments [51]. Using stable isotope tracing, it was found that In the absence of TGFβ, fibroblasts can maintain TCA cycle flux in low glutamine conditions by activating pyruvate carboxylase (PC) [51] (Figure 3A). In addition to pyruvate’s use via the PDH complex to generate acetyl-CoA, PC activity generates oxaloacetate from pyruvate and bicarbonate, thus allowing fibroblasts to use glucose-derived pyruvate as an anaplerotic substrate. This mechanism creates sufficient precursors for aspartate and glutamine *de novo* synthesis to maintain tRNA charging and translation in low glutamine conditions [51] (Figure 3A). The requirement for PC to maintain fibroblast collagen synthesis has been confirmed in breast and pancreatic xenograft tumors, with fibroblast PC also supporting tumor growth in these models [51]. In addition, PC-mediated anaplerosis is necessary for the expression of most collagen genes in low glutamine conditions [51]. By maintaining citrate and alpha-ketoglutarate levels, PC activity provides the substrates for modulation of histone acetylation and methylation [51] (Figure 3A). Why, then, can TGFβ-stimulated fibroblasts not use the

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same mechanism to maintain the TCA cycle, tRNA charging, and collagen gene expression in this environment? TGFβ signaling represses *PC* in an SMAD4-dependent fashion and inhibits PC activity [51]. Thus, TGFβ-induced glutamine dependency for anaplerosis is caused by the suppression of PC [51]. The finding that PC is conditionally essential for collagen synthesis in the low glutamine environments of breast and pancreatic tumors presents an opportunity to selectively target collagen production in these tumors.

Glucose and Lactate Availability—Glucose concentration is known to vary drastically in some pathologies. Hyperglycemia, as seen in conditions like diabetes, leads to an accumulation of collagen I and III through an increase in TGFβ production and MAPK signaling [89,90]. High glucose concentration also stimulates collagen I production via activation of Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling through a reactive oxygen species-mediated mechanism [91,92]. In contrast, complete depletion of glucose from the media impairs TGFβ-induced collagen I synthesis, which can be partially rescued by glycine supplementation [29,31], as discussed above. While these studies inform about the requirement of glucose for collagen synthesis, they do not adequately reflect the low glucose concentrations found in wounds and tumors, which are significantly lower compared to that of normal tissue and plasma but not zero [80,93,94]. When cultured in low compared to high glucose-containing media (2.8 mM v. 25 mM), the rate of collagen synthesis in fibroblasts is reduced, resulting in lower total collagen content [95,96]. To further model the metabolic environment of tumors, glucose levels were reduced in the context of low glutamine availability, which revealed a glucose concentration-dependent decrease in collagen I levels [51]. Collagen I depletion at 1–2 mM glucose and 0.2–0.4 mM glutamine was fully rescued by supplementation of 10–20 mM lactate [51], levels reflective of its concentration in the tumor microenvironment [86]. The ability of lactate to support collagen I synthesis under these conditions depends on uptake via monocarboxylate transporter 1 (MCT1), conversion to pyruvate via lactate dehydrogenase (LDH), and the use of lactate-derived pyruvate for anaplerosis via PC [51] (Figure 3A). Notably, lactate carbons directly contribute to hydroxyproline and, thus, collagen in the ECM in a PC-dependent fashion (Figure 3A), indicating that lactate can be recycled by fibroblast to fuel the TCA cycle, maintaining NEAA synthesis to support collagen production [51].

Lactate has been demonstrated to fuel mitochondrial metabolism in almost every tissue [97]. In addition to tumors, lactate concentration increases to 10–15 mM in healing wounds [85], and lactate accumulates in the lungs of IPF patients and mice treated with the fibrosis-inducing agent bleomycin [98,99]. Green and Goldberg were the first to demonstrate in 1964 in cultured fibroblasts that collagen synthesis almost doubles when extracellular lactate rises to 15 mM [100], a phenomenon shown later to be LDH-dependent [101]. In addition to maintaining NEAA synthesis as described above, lactate supplementation is thought to promote collagen gene expression at the epigenetic level. Lactate increases DNA hydroxymethylcytosine (5hmC) globally in fibroblasts, which decorates genes highly expressed in CAFs, including several collagens [102]. This effect is likely mediated by lactate's ability to increase alpha-ketoglutarate [102] (Figure 3A), a cofactor for dioxygenases catalyzing hydroxylation of the repressive 5-methylcytosine mark.

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4.2 Oxygen tension

Impaired perfusion in wounds, fibrotic tissue, and tumors reduces oxygen delivery, creating a hypoxic environment [87,103]. Oxygen availability is sensed by HIF-1α and HIF-2α, acting as transcription factors to regulate cellular adaptation to low-oxygen environments [103]. Therefore, the role of hypoxia in biology is often investigated through genetic modulation of HIF-α proteins. Despite the relevance of hypoxia, its role in regulating fibroblast collagen synthesis is not well understood, particularly from a metabolic perspective. Nevertheless, hypoxia is consistently reported to increase levels of functional, appropriately post-translationally modified collagen I in cultured fibroblasts by upregulating the expression of prolyl and lysine hydroxylases in a HIF-1α-dependent manner [104]. In chondrocytes, sustained HIF-1α activation downregulates collagen synthesis [105,106]. Conversely, it promotes collagen stability metabolically by increasing alpha-ketoglutarate levels, which supports collagen proline and lysine hydroxylation [105,106]. Hypoxia correlates with increased collagen I deposition in cardiac fibrosis [107], although it is unclear whether hypoxia is the cause or consequence of fibrosis. Consistently, fibroblast HIF-1α is required for bleomycin-induced pulmonary fibrosis [52]. However, as TGFβ induces transient HIF-1α stabilization via mitochondrial reactive oxygen species [24], it remains unclear whether the effects of HIF-1α deletion in fibroblasts reflect hypoxia or TGFβ biology. In addition, deletion of fibroblast HIF-1α reduced the number of αSMA$^+$ myofibroblasts in fibrotic lungs [52], which might contribute to reduced collagen abundance. This contrasts with breast tumors, where hypoxic regions are associated with reduced collagen I fiber density [108]. This likely reflects reduced collagen production by breast CAFs, which had lower intra- and extracellular collagen I protein levels when cultured in hypoxia [109]. Other collagen types were also differentially regulated in CAFs in hypoxia, including downregulation of collagen IV and upregulation of collagen VI [109], but the underlying mechanism is not understood. In addition to *COL1A1/2*, hypoxia suppresses *αSMA* expression and, thus, a myofibroblastic phenotype in CAFs in a HIF-1α-dependent fashion across tumor types [110,111]. Consistently, αSMA$^+$ CAFs were absent from hypoxic regions in breast and pancreatic tumors [110,111]. Notably, CAFs identified using a pan-fibroblast marker were still present in the hypoxic areas but adopted an inflammatory phenotype [111]. A hypoxia response signature was enriched in inflammatory CAFs across several tumor types [112], suggesting an important role of oxygen tension in regulating the fibroblast state. It is unclear how these differences between lung fibroblasts and CAFs in response to hypoxia arise, but it may involve tissue-specific differences in fibroblast biology. Moreover, given HIF-1α's role beyond hypoxia, investigating hypoxia through *HIF1α* deletion alone might not only adequately reflect hypoxia biology but rather a cellular stress response. Therefore, the role of hypoxia in regulating collagen synthesis in fibroblasts in different contexts warrants further investigation.

4.3 How fibroblasts integrate altered metabolite levels in tumors to sustain collagen synthesis

Microenvironmental alterations in glutamine, glucose, lactate, and oxygen in tumors and wounds result from limited perfusion and must, therefore, be considered in concert, not individually. Currently, no study has investigated how these alterations act in tandem to regulate fibroblast collagen synthesis. However, available data on concomitant glutamine

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and glucose reduction in the presence of lactate [51] suggest the following model (Figure 3A): extracellular lactate acts as the primary fuel for mitochondria, maintaining the TCA cycle via both PDH and PC upon conversion to pyruvate when glucose and glutamine levels decline. Lactate utilization for mitochondrial oxidation reduces glycolysis [113], freeing up the limited glucose and glycolytic carbon for preferential use in serine/glycose biosynthesis (Figure 3A). Similarly, lactate-mediated anaplerosis conserves glutamine-derived glutamate for proline biosynthesis (Figure 3A). In addition, some of the lactate carbon used for mitochondrial oxidation directly supports the proline synthesis for collagen translation [51] (Figure 3A). These data suggest that lactate utilization allows fibroblasts to decouple glycolysis and glutaminolysis from the TCA cycle. This allows their intermediates to be used preferentially in anabolic reactions that support collagen synthesis. We propose that future approaches to target collagen production in tumors selectively may exploit metabolic adaptations of CAFs imposed by the altered metabolic environment of tumors.

How does reduced oxygen tension fit into this model? Given the importance of mitochondrial oxidative metabolism for collagen synthesis described above, we expect hypoxia to suppress the biosynthetic reactions supporting collagen translation, leading to an overall decline in collagen production despite hypoxia's well-established role in promoting collagen stability at the posttranslational level. Findings in chondrocytes support this idea [105]. How fibroblasts integrate hypoxia with other metabolic stresses needs to be better understood because it is often studied in isolation. Conditions reflecting a concomitant reduction in oxygen and other blood-derived nutrients are rarely incorporated into studies investigating how hypoxia modulates collagen synthesis and fibroblast biology. Thus, to better understand the physiological role of hypoxia in regulating collagen synthesis, we call for hypoxia to be investigated in the proper metabolic context.

CAFs and their collagen are abundant in solid tumors such as breast and pancreatic cancer. At the same time, TGFβ-stimulated fibroblasts depend on environmental glutamine for collagen synthesis and, therefore, cannot produce collagen in the low glutamine environment found in breast and pancreatic tumors [51]. This creates a paradox: how can collagen be so abundant in such tumors if TGFβ-driven myofibroblastic CAFs, the primary collagen producers [16], are glutamine-dependent? The recent finding of metabolic heterogeneity in pancreatic tumors and that myofibroblastic CAFs are excluded from hypoxic tumor regions [111] might explain this. Hypoxic areas are likely also depleted in other blood-derived nutrients, such as glutamine. This suggests that myofibroblastic CAFs absent from such regions likely do not experience glutamine deprivation at a level that would impair their ability to produce collagen. Alternatively, CAFs residing in such areas of metabolic stress might change the kind of ECM they produce. Consistent with this idea, ECM produced by TGFβ-driven CAFs in low glutamine conditions is devoid of collagen but rich in fibronectin [51]. In addition, breast CAFs differentially alter the production of collagen types under hypoxia [109]. Based on these findings, we propose the existence of a nutrient-dictated matrisome (Figure 3B). This concept suggests that metabolic alterations during tumor progression differentially affect the ability of fibroblasts to produce various ECM molecules. While the synthesis of some ECM molecules might be more sensitive to fluctuating nutrients, others are relatively resistant, resulting in changes in ECM composition that will likely affect cell-matrix interactions. Notably, it has been suggested that the accumulation of

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fibroblast-derived ECM is a significant driver of impaired perfusion in tumors [12], placing the ECM at the beginning and the end of a vicious cycle that promotes tumor progression (Figure 3B).

5 Conclusions and Perspectives

Over the last decade, our understanding of how cellular metabolism and the metabolic environment regulate fibroblast collagen synthesis has been substantially enhanced. Conceptual and technological advances have enabled this remarkable progress. Conceptually, the realization that metabolism is not one size fits all but that different cell types and biological processes have distinct metabolic needs [114] suggests that fibroblasts engaging in collagen synthesis need different metabolic strategies to fulfill their specific demands compared to cancer cells and other highly proliferative normal cell types. Technologically, the increasing availability of metabolic assays beyond the cancer metabolism community, such as stable isotope tracing, has enabled other researchers to adopt these methods to study fibroblast biology. In combination with methods from the fibroblast and ECM community, including assays to produce fibroblast-derived, physiologically relevant ECM in culture [115], this creates a powerful toolbox for identifying metabolic pathways contributing to collagen synthesis. This liaison has led to the demonstration that fibroblasts not only use the major nutrients glucose and glutamine to synthesize free glycine and proline, respectively but that glucose-derived glycine and glutamine-derived proline are then incorporated into secreted collagen [24,29]. Combined, these advances have allowed the field to identify the primary nutrients and the metabolic pathways they are utilized in for fibroblasts to fulfill the metabolic demands of collagen synthesis. Concomitantly, these studies also identified several small molecule inhibitors targeting metabolic enzymes involved in collagen synthesis with efficacy in pre-clinical models of cancer and fibrosis. A major challenge for the field is translating these findings into clinically actionable strategies.

A critical step towards this goal is to define the relevance of identified metabolic pathways *in vivo* and in conditions that better reflect the physiological or pathological conditions studied. Advances in performing stable isotope tracing in animal models [116] will allow researchers to probe the contribution of various nutrients to fibroblast-derived collagen *in vivo*. Such experiments may confirm existing paradigms from *in vitro* studies, as demonstrated for the role of glutamine in synthesizing proline used for collagen I production in breast xenograft tumors [44]. They may also reveal novel nutrient sources or metabolic pathways utilized exclusively in an *in vivo* context, where multiple cell types interact, and the metabolites available to fibroblasts differ from traditional cell culture studies. Given that *in vivo* stable isotope tracing approaches can be technically challenging, the advent of physiological media [84] will allow researchers to conduct these experiments in an easily accessible, more controlled environment in metabolic conditions that better reflect those present *in vivo*. Implementing physiological media in cell culture models is an important next step in defining metabolic pathways that are physiologically relevant for collagen synthesis. In addition to validating established pathways, these studies will help identify physiologically relevant nutrients contributing to collagen synthesis that are not present in standard media, as demonstrated for lactate [51].

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The ECM generated by tissue-specific fibroblasts under physiologically relevant metabolic conditions may also better reflect the ECM composition of the respective tissue *in vivo*. The ability to engineer fibroblast-derived ECM under such conditions may enable researchers to better model *in vivo* relevant cell-matrix interactions that could otherwise only be observed with complex whole-tissue decellularization approaches. In this context, rigorously characterizing such matrices beyond the abundance of individual components, including biomechanical and biophysical parameters, will be necessary. How nutrient availability and utilization regulate these parameters is an open question. Similarly, most studies investigating the metabolic control of matrix synthesis have focused on collagen I, but how nutrient availability and utilization contribute to the production of other important ECM molecules remains to be investigated. Subsequently, it will be important to determine how fibroblasts allocate nutrients into different metabolic pathways to coordinate the synthesis of collagens and other ECM molecules. Such investigation might reveal essential determinants of ECM composition produced by various fibroblast types and other cells. Finally, the abundance of collagens and other ECM molecules is regulated by a balance of synthesis and degradation that skews towards synthesis in fibrotic disease. Beyond the reported role of fatty acid oxidation in promoting collagen degradation [56], how nutrient availability and utilization regulate ECM degradation is unknown and an upcoming area of investigation.

The role of metabolism in regulating collagen production is increasingly appreciated in other non-fibroblast mesenchymal and non-mesenchymal cell types. For example, macrophages can synthesize various collagens and use arginine-derived ornithine as a source of proline [117], which contrasts with glutamine's dominant role in proline synthesis in fibroblasts. Why these differences in nutrient choice exist remains unclear but may involve differential arginase expression and activity, which is enriched in the myeloid compartment [118]. Fibroblasts have been suggested to use ornithine derived from arginase activity in macrophages for proline biosynthesis [119], indicating that metabolic crosstalk between macrophages and fibroblasts could be a source of metabolites contributing to collagen synthesis that is currently not appreciated. Differences in nutrient preference also exist between mesenchymal cell types. Proline uptake via SLC38A2 and incorporation into collagen I increase during osteoblast differentiation, and osteoblasts preferentially use extracellular proline over glutamine-derived proline for protein synthesis [120]. Why osteoblasts prefer proline uptake compared to *de novo* synthesis during differentiation remains to be investigated. The underlying mechanism may be related to the fact that transcription factors necessary for osteogenic differentiation, including RUNX2, are also proline-rich [120], suggesting that proline availability may act as an additional rheostat, ensuring that cells have the proper resources to commit to the metabolically highly demanding process of differentiation.

Cancer cells also produce ECM proteins in tumors that are associated with poor prognosis in patients and promote pancreatic tumor progression [22,121]. Interestingly, pancreatic cancer cells produce the collagen I homotrimer consisting of three alpha-1 chains (α1/α1/α1) due to methylation of the *COL1A2* promoter, while fibroblasts produce the collagen I heterotrimer (α1/α2/α1) [122]. Whether altered metabolism in pancreatic cancer cells compared to CAFs contributes to the differential production of collagens and other ECM proteins remains to be investigated. Finally, the field has come to appreciate the substantial

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heterogeneity of fibroblasts in cancer, fibrosis, inflammation, and wound healing [123]. While myofibroblastic CAFs are considered the primary source of collagens in pancreatic tumors [16], gene expression data suggest that other pancreatic CAF types are also enriched for certain collagen types [124]. For example, inflammatory CAFs are selectively enriched for expression of *COL14A1* in pancreatic cancer [124,125]. What underlies the differential expression of collagen types in CAF subsets is unclear but may be related to their differential accumulation in metabolically discrete tumor regions [111]. Pancreatic inflammatory CAFs are enriched in hypoxic tumor regions [111], and hypoxia induces COL14A1 protein in murine lungs [126]. However, COL14A1 protein was downregulated in breast CAFs under hypoxia [109], indicating additional tissue-specific regulation.

In conclusion, while much has been learned regarding the metabolic control of collagen synthesis over the last few years, much remains left to be uncovered (Box 1). Now that the pathways toward some of the arguably more obvious key metabolites for collagen synthesis have been described, the field is challenged with demystifying nutrients and metabolic pathways with no apparent link to collagen. Adopting paradigms and technologies from cancer- and immunometabolism will help advance the ECM community in unraveling the metabolic networks underlying fibrotic responses. A deeper understanding of how these networks support ECM synthesis and maintenance will reveal novel aspects of ECM biology, providing opportunities to develop therapeutic strategies that benefit patients with cancer or fibrotic disease.

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Highlights

- ECM synthesis poses significant demands for biomass generation with requirements different from cell proliferation.
- With its high glycine and proline content, collagen synthesis imposes unique biosynthetic demands
- Pro-fibrotic signaling cascades mediate metabolic rewiring in activated fibroblasts
- Targeting the metabolic pathways supporting collagen synthesis provides opportunities for treating fibrotic conditions.
- Nutrient availability influences metabolic pathways regulating collagen synthesis and creates new vulnerabilities.

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Box 1: Open questions

Specific

- How do fibroblasts decide whether to allocate glutamate for anaplerosis or proline biosynthesis?
- What is the relative contribution of mitochondrial NADPH *de novo* synthesis v. regeneration in proline and serine/glycine biosynthesis?
- How do fibroblasts generate sufficient hypusinated eIF5A for efficient collagen translation?

General

- How is collagen degradation regulated metabolically?
- Why do non-fibroblast collagen producing cells use different nutrients to produce collagen?
- Why do different fibroblast subtypes express different collagen types? Does synthesis of different collagen types require similar metabolic processes?
- How do fibroblasts integrate hypoxia with other metabolic stresses to regulate collagen production?
- How do fibroblasts allocate nutrients into different metabolic pathways to produce all ECM molecules appropriately?
- How does nutrient availability and metabolic pathways act on a network level to support the metabolic demands of activated fibroblasts?
- How does nutrient availability affect the matrisome?
- What are the nutrients and metabolic pathways that are physiologically relevant for collagen synthesis?
- Are there tissue-specific metabolic programs that are relevant to collagen synthesis?
- Is metabolic reprogramming clinically actionable to impair fibrotic disease? Can it be targeted to inhibit fibrotic responses in a tissue- or disease-specific fashion?

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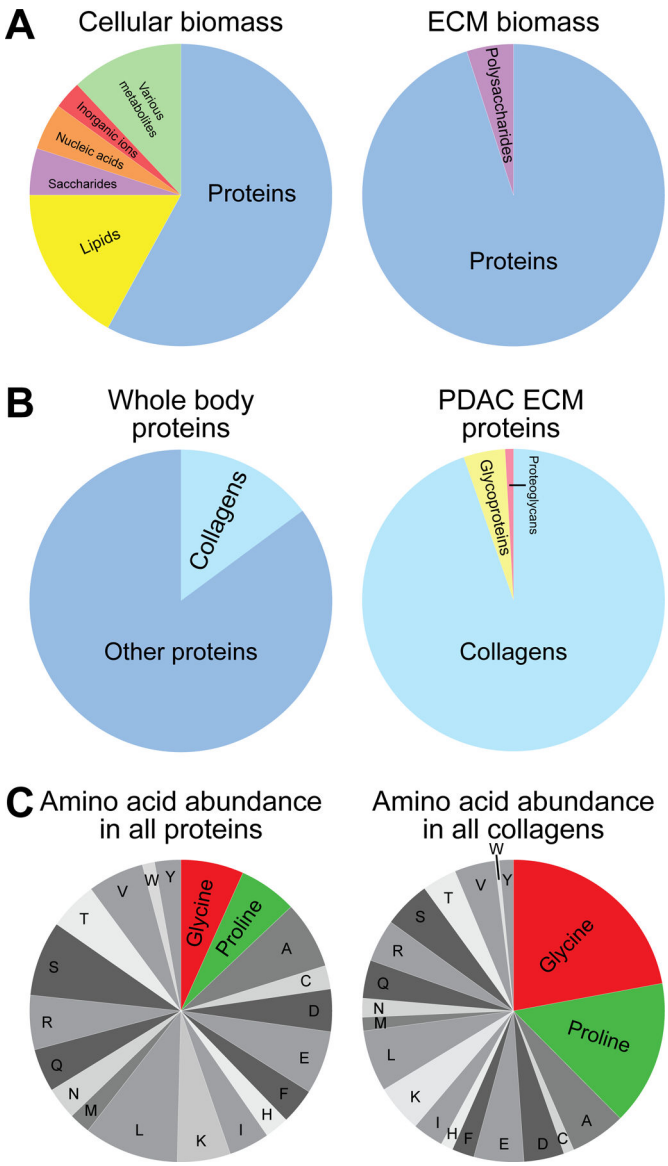

Figure 1: Biochemical composition and amino acid profiles of cellular and extracellular matrix (ECM) biomass.

(A) Left: composition of cell dry mass, adapted from [19]. Right: composition of ECM dry mass, based on [20].

(B) Left: relative whole-body collagen content in mice, based on [21]. Right: human pancreatic ductal adenocarcinoma (PDAC) ECM protein composition, based on [22].

(C) Comparison of amino acid abundance in all human proteins (left) versus all collagens (right). Based on data from the NCBI protein database with the assumption that each protein has the same abundance. Letters represent amino acid letter codes.

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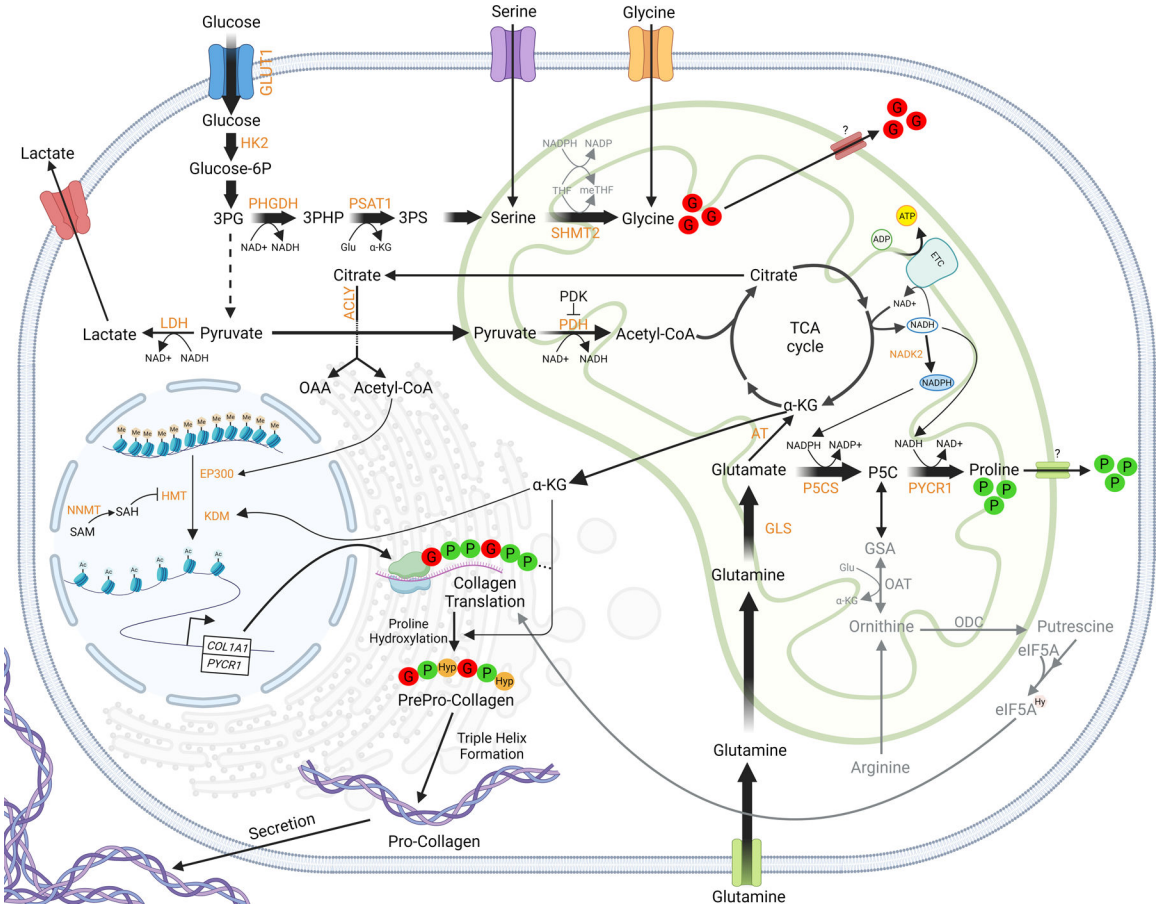

Figure 2: Metabolic rewiring that promotes collagen synthesis in fibroblasts.

In a pro-fibrotic environment, fibroblasts increase glucose and glutamine uptake to sustain the epigenetic, bioenergetic, biosynthetic, and post-translational requirements of collagen synthesis. Thick arrows indicate the pathway fluxes that TGFβ stimulation or the CAF state induces. Metabolites, enzymes, and arrows in grey denote proposed pathways that have not yet been experimentally verified. Question marks denote unknowns.

3PG: 3-Phosphoglyceric acid; 3PHP: 3-phosphohydroxypyruvate; 3PS: 3-phospho-L-serine; αKG: alpha-ketoglutarate; Ac: acetyl; ACLY: ATP citrate lyase; AT: aminotransferase; ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; eIF5A: eukaryotic translation initiation factor 5A; EP300: Histone acetyltransferase p300; ETC: Electron transport chain; G: glycine; Glu: glutamate; GLUT1: Glucose transporter 1; GLS: Glutaminase; GSA: Glutamate-γ-semialdehyde; Hy: Hypusine; Hyp: Hydroxyproline; HK2: Hexokinase 2; HMT: Histone methyltransferases; KDM: Histone lysine demethylase; LDH: Lactate dehydrogenase; Me: methyl; mTHF: Methyltetrahydrofolate; NAD: Nicotinamide adenine dinucleotide; NADK2: Nicotinamide adenine dinucleotide kinase 2; NNMT: nicotinamide N-methyltransferase; OAA: Oxaloacetate; OAT: Ornithine aminotransferase; ODC: Ornithine decarboxylase P: proline; P5CS: Pyrroline-5-carboxylate synthase; PDH: Pyruvate Dehydrogenase; PDK: Pyruvate Dehydrogenase kinase; PHGDH: phosphoglycerate dehydrogenase; PSAT1: phosphoserine aminotransferase 1; PYCR1: Pyrroline-5-carboxylate reductase 1; SAM: S-adenosyl-methionine; SAH: S-

adenosylhomocysteine; SHMT2: serine hydroxymethyltransferase 2; TCA: tricarboxylic acid; THF: Tetrahydrofolate.

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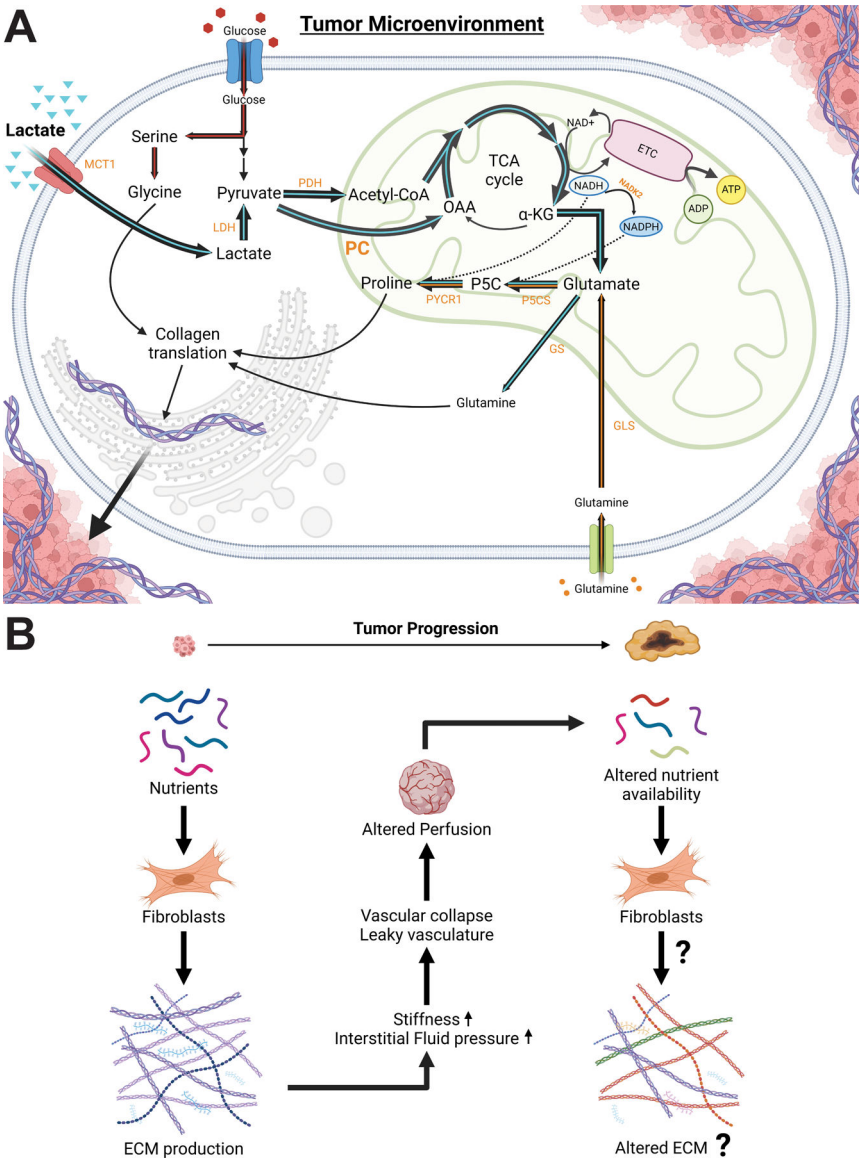

Figure 3: Metabolic adaptations in the tumor microenvironment (TME).

(A) Metabolic rewiring of fibroblasts: In the TME, glutamine and glucose are scarce, whereas lactate is abundant. Metabolic fluxes for collagen synthesis are altered compared to standard culture conditions (Figure 2). Lactate-derived pyruvate is used via PDH and PC to maintain the TCA cycle and replenish glutamate pools. This maintains tRNA charging through *de novo* glutamine synthesis and supports proline biosynthesis. Lactate utilization also allows the remaining glutamine and glucose to be used efficiently for proline and glycine biosynthesis, respectively. Colored lines represent the flux of nutrients inside the cell. In blue: lactate, in red: glucose, in orange: glutamine.

αKG: alpha-ketoglutarate; ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; GLS: Glutaminase; GS: Glutamine synthetase; LDH: Lactate dehydrogenase; MCT1: Monocarboxylate transporter 1; NAD: Nicotinamide adenine dinucleotide; NADK2: Nicotinamide adenine dinucleotide kinase 2; OAA: Oxaloacetate; P5CS: Pyrroline-5-

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carboxylate synthase; PC: Pyruvate carboxylase; PYCR1: Pyrroline-5-carboxylate reductase 1; TCA: tricarboxylic acid.

(B) The nutrient-dictated extracellular matrix (ECM): during tumorigenesis, nutrients are used by fibroblasts to produce ECM. The ECM, when in excess, alters the biomechanical and biophysical properties of the TME, causing reduced perfusion. This alters nutrient availability for cancer-associated fibroblasts (CAFs). We propose that CAFs produce an ECM that is different in quantity and quality in such altered nutrient conditions: the nutrient-dictated extracellular matrix.